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Treatment with a Copper-Zinc Chelator Markedly and Rapidly Inhibits β-Amyloid Accumulation in Alzheimer's Disease Transgenic Mice

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Summary

Inhibition of neocortical β-amyloid (Aβ) accumulation may be essential in an effective therapeutic intervention for Alzheimer's disease (AD). Cu and Zn are enriched in Aβ deposits in AD, which are solubilized by Cu/Zn-selective chelators in vitro. Here we report a 49% decrease in brain Aβ deposition (−375 μg/g wet weight, p = 0.0001) in a blinded study of APP2576 transgenic mice treated orally for 9 weeks with clioquinol, an antibiotic and bioavailabl Cu/Zn chelator. This wa accompanied by a modest increase in soluble Aβ (1.45% of total cer bral Aβ); APP, synaptophysin, and GFAP levels were unaffected. General health and body weight parameters were significantly more stable in the treated animals. These results support targeting the interactions of Cu and Zn with Aβ as a novel therapy for the prevention and treatment of AD.

Introduction

β-amyloid peptide (Aβ), which accumulates in the neocortex in Alzheimer's disease (AD), possesses selective high- and low-affinity Cu^{2+} and Zn^{2+} binding sites that mediate both its protease resistance, reversible precipitation (Atwood et al., 1998, 2000; Bush et al., 1994a, 1994b; Huang et al., 1997), as well as the O_2-dependent production of H_2O_2 (Aβ42 > Aβ40) and concomitant toxicity (Cuajungco et al., 2000; Huang et al., 1999a, 1999b). Cu and Zn are elevated in the neocortex in AD and particularly concentrated in amyloid plaques (Lovell et al., 1998; Suh et al., 2000). We recently reported that Cu/Zn chelators solubilize Aβ from postmortem AD brain tissue (Cherny et al., 1999). Recent studies of amyloid deposits in the APP2576 transgenic (Tg) mouse model of AD (Hsiao et al., 1996) have identified enrichments of Zn (Lee et al., 1999) and Fe (Smith et al., 1997), resembling those seen in AD amyloid (Cu levels have not yet been studied in this model). Therefore, we sought to determine whether treatment with a bioavailable chelator would inhibit brain β-amyloid deposition in this Tg mouse model.

In order to identify agents for testing, we first considered existing US Pharmacopoeia (USP) drugs with established toxicology profiles, so that the initiation of clinical trials could be accelerated. Chelators such as triene (TETA), penicillamine, and desferrioxamine are safely used pharmacologically for the treatment of metal overload disorders, such as Wilson's disease. However, these molecules are hydrophilic and exert their effects by systemic depletion of metals, and do not pass across the blood-brain barrier. Hence, while we have established that these common chelating compounds do reverse metal-induced Aβ aggregation and H_2O_2 production (Atwood et al., 1998; Bush et al., 1999; Huang et al., 1997, 1999a, 1999b), they are unlikely to penetrate the brain Aβ mass in AD mouse models. There are many other USP drugs that, while not being termed chelators, have chelating properties and favorable toxicity profiles. This is generally true of the quinoline and quinolone drug class. One example is clioquinol (CQ, iodochlorhydroxyquin, 5-chloro-7-iodo-8-hydroxyquinoline, MW = 305.5), a quinoline that selectively binds Zn^{2+} and Cu^{2+} with greater affinity than it binds Ca^{2+} and Mg^{2+} [$K_1(Zn)$ = 7.0, $K_1(Cu)$ = 8.9, $K_1(Ca)$ = 4.9, $K_1(Mg)$ = 5.0]. CQ is hydrophobic and freely crosses the blood-brain barrier (Padmanabhan et al., 1989). It theref re possesses some of the ideal prototypic properties for a candidate agent

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that could solubilize Zn/Cu-assembled Aβ deposits in viv and inhibit Aβ redox chemistry. CQ was used extensiv ly as an oral antibiotic (Richards, 1971) before it was withdrawn in the early 1970s du to overdose-associated neurological sid effects that are now beli ved to be preventable with B12 supplementation (Yassin t al., 2000).

Her w report the effects of CQ (oral treatment) on aged APP2576 Tg mice with advanced Aβ deposition. CQ treatment for 9 weeks markedly inhibited cerebral Aβ deposition by 375 μg/g wet weight compared to sham-treated controls. These changes were accompanied by no adverse effects and a significant improvement in scores on a general behavior rating scale. These findings are strong support for the role of zinc and copper interaction with Aβ in the pathophysiology of AD and indicate that the CQ class of agents could have therapeutic utility in AD.

Results

We first studied CQ in filtration assay systems that we previously used to identify several chelators that inhibit and reverse Zn/Cu-induced aggregation of synthetic Aβ1-40 and Aβ1-42 peptides in vitro (Atwood et al., 1998, 2000; Bush et al., 1994a, 1994b; Huang et al., 1997; Moir et al., 1999). CQ (2 μM, in TBS, pH 7.4) was significantly more effective than EDTA (2 μM, in TBS, pH 7.4) in dissolving Aβ1-40 aggregates induced by either Zn^{2+} or Cu^{2+} (Figure 1A). Neither chelator could resolubilize Aβ1-40 aggregates induced by incubating the peptide at pH 5.5, which induces β-sheet formation (Wood et al., 1996) (Figure 1A).

Potential binding interactions of CQ with Aβ were studied by NMR spectroscopy of Aβ1-28, which possesses the metal binding sites of Aβ centered around the 3 histidine residues (Atwood et al., 1998; Bush et al., 1994a), as well as a domain (residues 17-21) that binds to peptide fibrilization inhibitors (Soto et al., 1996). When one equivalent of Cu^{2+} was added to Aβ1-28 (1 mM in aqueous solution), the metal bound to the histidine residues as evidenced by broadened NMR peaks observed in the differences between the spectra shown in Figure 1B, scans A and B. The addition of CQ to this solution restored the peaks that had been broadened in the starting NMR spectrum of Aβ (Figure 1B, scan C) due to the presence of copper. This result is consistent with CQ removing bound copper from Aβ. There were no changes to the NMR spectrum of Aβ1-28 upon additi n of CQ alone, and addition of CQ to either aqueous or DMSO solutions of Aβ1-40 (0.3 mM) did not affect the NMR spectrum (data not shown), suggesting that CQ does not act as a fibril inhibitor by binding the peptide. NMR spectra of CQ alone were recorded and found not to be perturbed by the addition of Aβ1-40 (0.3 mM), confirming that CQ does not bind to the peptide.

In order to confirm that CQ, like other Cu^{2+}/Zn^{2+}-selectiv chelators, could chemically solubilize Aβ deposits in AD (Cherny et al., 1999), we homogenized postmortem human brain samples affected by AD in the presence of CQ. W found that there was a concentration-dependent increase in Aβ liberated into th soluble phase, typically >200% in the presence of ≥0.4 μM CQ (Figure

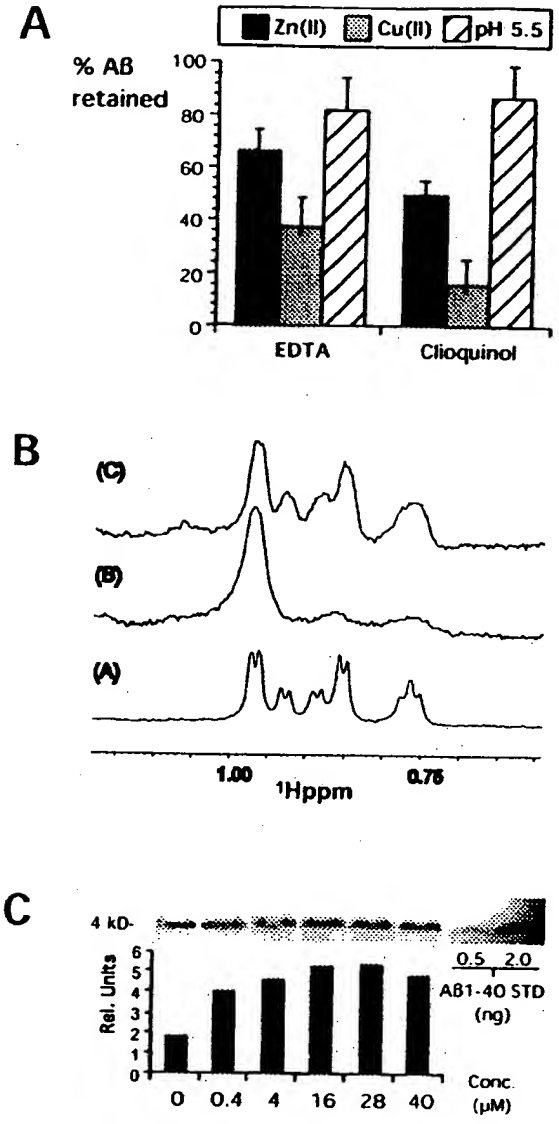

Figure 1. Interactions of Clioquinol with Aβ In Vitro

(A) Effects of CQ upon the retention of Aβ1-40 aggregates induced by Zn^{2+}, Cu^{2+}, or pH 5.5. The procedure was a modification of one which we have previously reported (Moir et al., 1999). Values are expressed as a percentage of the amount of aggregated Aβ detected after washing with TBS pH 7.4 vehicle alone (100%), represented as mean ± SD, n = 3.

(B) NMR spectroscopy of Aβ1-28 in the presence of Cu^{2+} and CQ. *A*, Spectrum of Aβ1-28 in saline buffer (pH 6.9) showing the peaks due to the methyl groups of Val-12, -18, -24 and Leu-17. *B*, Spectrum of sample in *A* after adding Cu^{2+}. *C*, Spectrum of the sample in *B* after adding CQ. The lower resolution of the spectrum in *C* is due to the presence of aggregated material in the sample.

(C) Enhanced extraction of Aβ from human AD-affected postmortem brain tissue following homogenization in the presence of clioquinol. The upper panel shows a Western blot (using WO2, which detects both Aβ1-40 and Aβ1-42) of the soluble fraction of frontal lobe, the same sample of which had been divided and homogenized in the presence of increasing concentrations of CQ (in PBS, pH 7.4). Corresponding densitometric quantification is shown in the lower panel. The data are representative of n = 9 AD cases.

1C, typical of 9 cases). The Aβ liberated by treatment with CQ was detected using monoclonal antibody WO2 (which d tects Aβ40 and Aβ42 at an epitope between residues 5–8, Figure 1C), and a similar proportional increase in Aβ40 and Aβ42 species compared to th baseline amounts xtracted by PBS was determined by blotting with G210 (specific for Aβx-40) and G211 (specific for Aβx-42) (Ida et al., 1996) (data not shown). The effect of CQ in enhancing Aβ liberation in this assay is comparable to the effect we previously reported in the same system for bathocuproine, a Cu chelator (Cherny et al., 1999). Similar to the effects of homogenizing the brain sample with other Cu/Zn-selective chelators (Cherny et al., 1999), the majority (90%) of the Aβ remains in the pellet phase after one extraction with CQ. However, repeated extractions continue to liberate approximately the same proportion of Aβ so that eventually the majority of the Aβ in the tissue is solubilized (data not shown).

In view of these results, we performed a pilot study of CQ treatment in the APP2576 Tg mice (Hsiao et al., 1996). We first compared the effects of CQ and triethylene tetramine (TETA, a hydrophilic Cu/Zn-selective chelator) on a cohort of 12-month-old APP2576 mice. The drugs were delivered by gavage daily for 12 weeks. The animals were sacrificed and brain Aβ levels were appraised. There was a mean decrease in the pellet fraction of cerebral homogenates from the animals treated with CQ 2 mg/kg/d group (275 ± 38 μg/g protein, n = 6) that did not reach statistical significance compared to sham-treated controls (316 ± 81 μg/g protein, n = 6). However, there was a significant 65% decrease in the levels of sedimentable Aβ in the mice treated with CQ 20 mg/kg/d (110 ± 56 μg/g protein, mean ± SE, n = 5, $p < 0.01$). Intriguingly, two animals in the CQ 20 mg/kg/d treatment group were found to have no measurable Aβ in the brain pellet fractions and no detectable amyloid pathology in their neocortex or cortical blood vessels (Figure 2). Transgenic status and the overexpression of APP were reconfirmed in all animals. TETA at 18 mg/kg/d had no significant effect on sedimentable Aβ levels either alone (494 ± 56 μg/g protein, n = 5) or in combination with CQ 2 mg/kg/d (342 ± 114 μg/g protein, n = 5).

To further test whether the amyloid-clearing effects of CQ, we next studied the effects of CQ at a higher dose (30 mg/kg/d) in a larger cohort (n = 20 on drug, n = 19 sham-fed transgenic controls) of older (21 months) APP2576 mice using more detailed analysis. We utilized a similar blinded protocol, in which the animals were administered CQ by daily gavage for a shorter interval (9 weeks), since the animals were of advanced age. There were similar numbers of male and female mice in each treatment group after randomization. Measurement of cerebral Aβ levels at the completion of the study indicated that there was again a marked and significant decrease in pellet Aβ in CQ-treated mice. The levels of pellet Aβ in sham-treated mice were 7.48 ± 0.73 mg/g protein, 770.0 ± 68.6 μg/g wet weight, and in CQ-treated mice were 4.44 ± 0.36 mg/g protein [41% decrease, $p = 0.001$], 394.6 ± 39.6 μg/g wet weight [49% decrease, $p = 0.0001$] (Figure 3A). The difference in sedimentable Aβ f ~375 μg/g wet weight of cerebral tissue after only 9 weeks of treatment with CQ indicat s a profound alteration in the rate of Aβ accumulation, which can be appreciated when contrasted t the average levels of Aβ in the pellet fraction of AD-affected cortical tissu (20 μg/g w t weight) (McLean t al., 1999) measured by the same technique.

Quantification of soluble cerebral Aβ l vels in the samples revealed a significant increase in the levels of soluble Aβ in th brains f the CQ-treated mice (0.25 ± 0.02 mg/g protein [+52%, $p = 0.004$], 8.06 ± 0.81 μg/g wet weight [+44%, $p = 0.014$]) compared to sham-treated controls (0.16 ± 0.01 mg/g protein, 5.61 ± 0.41 μg/g wet weight) (Figure 3B). This rise represented a small (1%) increase in the contribution of soluble Aβ to total cerebral Aβ content (CQ-treated 2.10% ± 0.19%, sham-treated 0.81% ± 0.09%; Figure 3C). However, the increase is soluble Aβ levels is very modest (0.6 μM, assuming one g wet weight = 1 ml) compared to th profound decrease in sedimentable Aβ (approximately −90 μM) in the cerebrum in the CQ-treated mice. Solubl APP (sham, 31.9 ± 2.4 μg/g wet weight; CQ, 34.9 ± 2.5 μg/g wet weight; $p = 0.41$) and pellet APP (sham, 138.2 ± 19.8 μg/g wet weight; CQ, 172.8 ± 21.4 μg/g wet weight; $p = 0.25$) levels in these samples were not significantly different in the CQ compared to the sham-treated cohorts, indicating that the decrease in total Aβ levels induced by CQ was not due to decreased APP production.

Accompanying these changes was a significant decrease ($p = 0.04$) in the immunohistochemical amyloid plaque surface area in the CQ-treated mice (13.0 ± 1.5 $\mu^2/100\ \mu^2$) compared to the sham-treated mice (17.3 ± 1.3 $\mu^2/100\ \mu^2$) (Figure 3D). There were no correlations between the levels of soluble Aβ, total Aβ, soluble/total Aβ ratio, or plaque surface area in either the CQ-treated mice or sham-treated mice (or combined groups), which is in agreement with our previous findings of a lack f relationship between soluble Aβ, sedimentable Aβ, and plaque surface area in postmortem AD brain specimens (McLean et al., 1999).

As a marker of synaptic loss, cerebral synaptophysin levels were assayed and determined to be unaffected (sham = 788 ± 102 U/g protein, n = 14; CQ = 720 ± 63 U/g protein, n = 13, $p = 0.57$). There was also an insignificant 20% decrease in hippocampal cells staining positively for glial fibrillary acidic protein (GFAP) in the CQ-treated mice (sham = 27.0 ± 4.3 cells/hpf, n = 13; CQ = 21.6 ± 3.7, n = 14, cells/hpf, $p = 0.34$). The brain masses and protein concentrations were unchanged in the CQ-treated mice compared to the sham-treated controls (sham = 0.262 ± 0.015 g wet weight/hemisphere, 23.42 ± 1.08 mg protein/hemisphere, n = 14; CQ = 0.279 ± 0.005 g wet weight/hemisphere, 20.95 ± 1.17 mg protein/hemisphere, n = 14, $p > 0.1$). No significant correlations were observed between synaptophysin or GFAP levels and levels of Aβ, plaque, or total protein.

Serum levels of Aβ were significantly decreased (−24%, $p = 0.04$) in the CQ-treated animals (115 ± 8 ng/ml) compared to sham-treated controls (152 ± 18 ng/ml) (Figure 3E). There was a significant correlation ($R^2 = 0.2$, $p = 0.03$) between serum Aβ levels and total cerebral Aβ levels of the combined groups.

To determine th ffects of treatment with CQ, we also measured metal levels (Al, Co, Cr, Cu, Fe, Mn, Pb, Se, Zn) in the solubl and sedimented fractions of brain

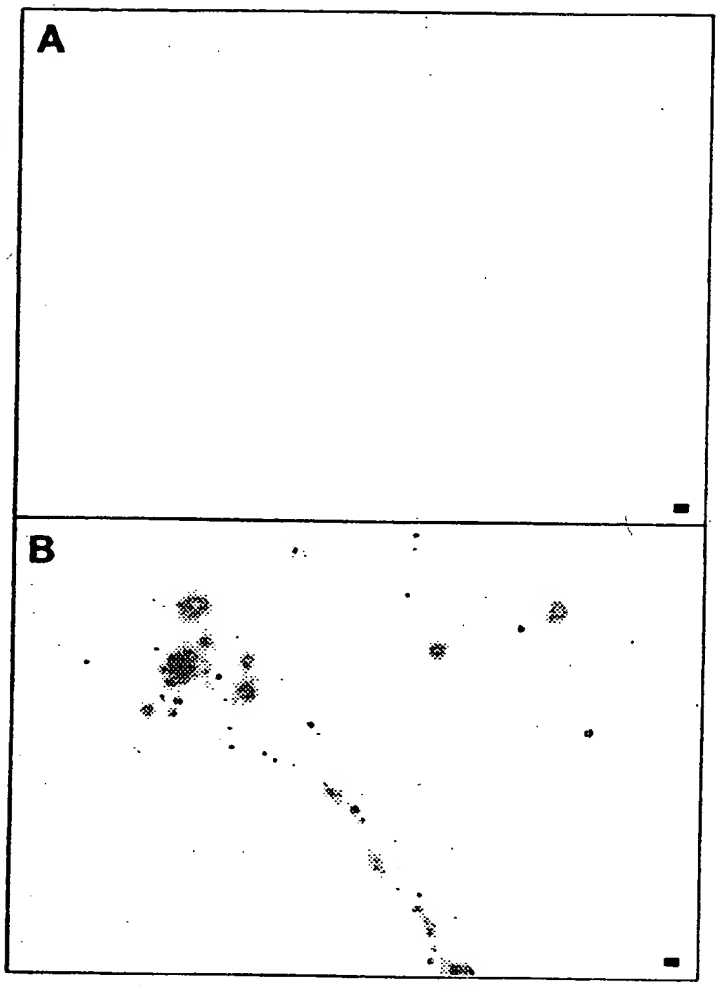

Figure 2. Initial Study of the Effects of Oral Treatment of 15-Month-Old APP2576 Transgenic Mice with Clioquinol

Immunohistochemistry of Aβ deposits in the hippocampal region of two 15-month-old APP2576 mice treated with either CQ 20 mg/kg/day (A) (representative of two animals) or sham-treated (B) (representative of three animals). The figure is typical of 4 sections analyzed throughout each brain. Size bar = 50 μm.

and peripheral organ homogenates from the 21-month-old APP2576 study cohort. We detected significant increases in Cu (+19%, +4.7 μM, $p = 0.007$) and Zn (+13%, +9.6 μM, $p = 0.006$) levels in the soluble cerebral fractions of the CQ-treated mice (Table 1), but no changes for any of the other metals measured. There was no significant change in any metal levels in the xtracted centrifugation pellet fraction of the cerebral homogenates of the CQ-treated mice. There was a significant 24% increase in Co in the soluble fraction of the liver homogenates of the CQ-treated mice, as well as significant 15% increase in Zn content of the kidney pellet fraction. There were no other differences in metal levels in the liver and kidney samples from the CQ compared to the sham-treated animals. There were also no significant correlations between the levels or ratios of the various metals with the levels or ratios of the Aβ lev ls in the soluble and sedimented brain fractions, or with the plaque surface area. There was a significant linear correlation between Cu and Zn levels in the cerebral fractions of the combined (CQ + sham) cohort ($R^2 = 0.2$, $p = 0.03$).

As an appraisal of th potential toxicity of CQ, we reviewed the vital data of the two cohorts of 21-month-old APP2576 mice. Weight measurements of the sham-treated and CQ-treated mic taken at intervals throughout the study were not significantly different until day 53 of the study, when it observed that the mice treated with CQ maintained their weight, whereas the weights of the sham-treated animals declined so that the surviving CQ-treated mice became significantly heavier (37.41 ± 5.09 g) than the sham-treated mice (33.19 ± 4.05 g, $p < 0.05$) at day 53 (Figure 4A). CQ did not significantly affect the longevity of the mice, as the mean survival intervals of the CQ-treated mice (53.8 ± 4.3 d) and the sham-treated mice (57.2 ± 2.9 d) as well as survival curves (Figure 4B) were not significantly different (log rank survival distribution = 0.35, $p = 0.55$). Therefore, there was no gross evidence of toxicity for CQ treatment at this dose.

Because of the advanced age of these animals, water maze testing was not possible. However, to gauge gross physiological changes caused by CQ on the treated mice, we devised a 5 point integer scale that subjectively rated a combination of general features (motor activity, alertness, and general health signs) and was administered by a blinded operator every day to each individual mouse. There was a decline in the readings of the sham-treated mice that plateaued after 16 days f treatment (Figure 4C), which may have been due to repeated handling of the animals. In contrast, after the same initial decline as the control mice, the CQ-treated mice then

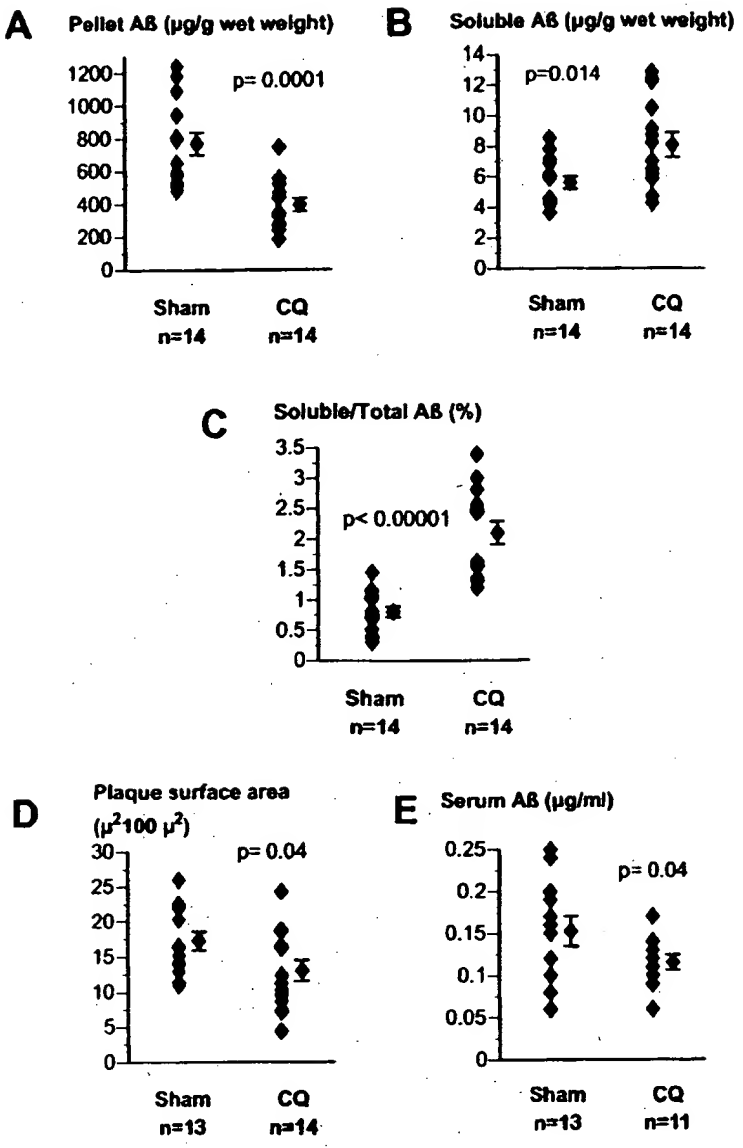

Figure 3. Effects of Oral Clioquinol Treatment on Aβ Metabolism in 21-Month-Old APP2576 Mice

(A and B) (A) Total and (B) soluble Aβ levels from cerebral homogenates of sham-treated mice and mice treated with clioquinol (30 mg/kg/day, CQ).

(C) Proportion of soluble Aβ compared to total Aβ (in percentage of mg/g wet weight values) in sham-treated compared to CQ-treated mice.

(D) Immunohistochemical plaque surface area from fixed cortical tissue of sham-treated mice and CQ-treated mice.

(E) Serum Aβ levels in sham-treated compared to CQ-treated mice.

recovered after 16 days, and their readings plateaued at a consistently higher mean score on this index than those of the sham-treated mice. This apparent benefit of CQ treatment was sustained from day 17 for each of the 46 remaining days of the study.

The plateauing in mean scores for each group follow-

Table 1. Effects of Oral Clioquinol Treatment on Distribution of Metals within Selected Tissues of 21-Month-Old APP2576 Mice

	Co (ng/g)		Cu		Zn	
	C	CQ	C	CQ	C	CQ
Brain (sol.)	18.1	12.3	1.70	**2.02****	4.83	**5.45****
Brain (pel.)	18.6	15.9	1.32	1.39	3.86	4.25
Liver (sol.)	27.1	**35.7***	3.26	3.41	29.6	34.0
Liver (pel.)	34.6	35.4	3.05	2.95	14.1	15.7
Kidney (sol.)	74.5	91.6	0.73	0.82	6.61	7.30
Kidney (pel.)	66.4	62.9	2.25	2.34	7.96	**9.12***

Metal levels (averages in ng or µg/g wet weight) in tissues where significant differences are noted between the C and CQ groups. Two-tailed t tests were performed on differences between the mean values from the sham-treated (C) compared to clioquinol-treated (CQ) samples. Significant differences in the mean values are represented by data in bold; asterisk, $p < 0.05$, and double asterisk, $p < 0.01$. No significant differences were found for levels of Al, Cr, Fe, Mn, Pb, or Se.

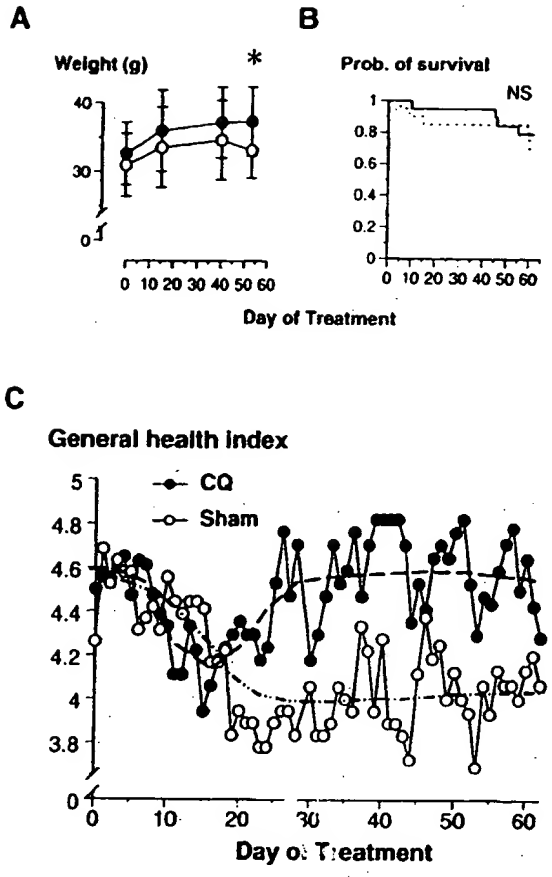

Figure 4. Effects of Clioquinol Treatment on the Systemic Health of 21-Month-Old APP2576 Mice

Parameters measured over the duration of the study: (A) weight (mean ± SD; asterisk, significant difference in mean weights in each group greater than the difference at day 0, $p < 0.05$; solid symbols are CQ treated); (B) survival curves (dashed is CQ treated); (C) general impairment (blinded subjective rating scale). Mean daily scores (±SEM) for the CQ-treated and sham-treated groups are indicated.

ing day 16 in Figure 3C is influenced by the integer scoring and nonlinear nature of the rating scale. A further breakdown of the daily scores (n = 746 individual observations) for CQ-treated group from day 17 onward revealed that 60.7% of the scores were 5 (out of 5, meaning no apparent impairment), 32.0% were 4 (meaning minor signs of impairment), 6.6% were 3 (meaning periodic signs of serious impairment), 0.7% were 2 (meaning persistent signs of serious impairment), 0.0% were 1 (meaning moribund). In contrast, the statistical mode for the sham-treated group was decreased to 4, with only 30.6% of daily readings being 5, 48.4% were 4, 12.6% were 3, 7.0% were 2, and 1.3% were 1 (n = 767 observations). Therefore, compared to sham-treated animals, treatment with CQ doubled the incidence of animals that appeared to be grossly normal (sham-treated = 30.6% of observations versus CQ-treated = 60.7%). Readings of 3 or less were relatively uncommon because once th animals became serious impaired, they usually died soon after. A two-way ANOVA with repeated measures on these results indicated that th improvement in the surviving CQ-treated mic was significant [$F(61, 1525) = 2.4$, $p < 0.0001$; observations included from day 0 until completi n]. Although this scale is n t a linear gaug f det rioration, the differences between the integ r scores in the treated and untreated groups support th conclusion that CQ induces a conspicuous improvement in the health of the transgenic mice.

Discussion

Taken together, our findings indicate that CQ treatment, for as little as 9 weeks, inhibits and possibly reverses accumulation of Aβ deposits in APP2576 transgenic animals. Our in vitro findings that CQ reverses Cu^{2+}- and Zn^{2+}-induced Aβ aggregates (Figure 1A) and, at concentrations as low as 400 nM, solubilizes Aβ deposits in AD-affected postmortem brain tissue (Figure 1C) support the likelihood that the in vivo effect we observed was due to interdiction of the interaction of these metal ions with cerebral Aβ. This likelihood is further supported by the observation that CQ complexes with Zn^{2+} in the brain (Shiraki, 1979), especially in areas enriched in synaptic vesicular zinc such as the temporal lobe, which is severely affected by amyloid deposition. The alternative possibility of CQ acting as a fibril chain-breaker was not supported by NMR spectroscopy (Figure 1B).

The possibility that CQ exerted its effects by chelating $Fe^{2+/3+}$ (Kidani et al., 1974) cannot be excluded, since $Fe^{2+/3+}$ precipitates Aβ (less effectively than Zn^{2+} or Cu^{2+}) (Atwood et al., 1998) and is also found to be enriched in plaque (Lovell et al., 1998). But unlike the Zn and Cu levels in the brain, treatment with CQ did not alter Fe levels in the APP2576 cohort. Also, chelators that solubilize Aβ from postmortem AD cortical specimens appear to redistribute Zn and Cu, but not Fe (Cherny et al., 1999). It is unlikely that the decrease in Aβ accumulation was due to decreased Aβ synthesis caused by CQ-associated toxicity, since there was no decrease in brain APP levels, brain synaptophysin levels were not decreased, and the CQ-treated mice exhibited signs of improved general health rather than signs of toxicity (Figure 4C).

TETA did not inhibit Aβ deposition in this animal model. This may be because, unlike CQ, it is not a lipophilic molecule, and therefore may not be able to penetrate the Aβ deposits, or because the dose was insufficient. We have observed, however, that higher doses of TETA (40 mg/kg/d) were rapidly toxic in non-Tg mice, therefore limiting its testing. In contrast, CQ is rapidly absorbed from the rodent gut with blood levels reaching 1–10 μM within 1 hr of ingestion (Kotaki et al., 1983), and since it is hydrophobic, it passes rapidly into the brain. CQ is rapidly excreted in the urine so that a bolus dose of clioquinol is almost completely removed from the brain within 3 hr (Toyokura et al., 1975).

Our results indicate that the beneficial effects of CQ treatment contrast favorably with the popular Aβ vaccination experimental treatment approach and with any of th other reported candidate AD treatments tested in adult transgenic mice. Schenk et al. (1999) reported that total cerebral Aβ (including Aβ1-40 and Aβ1-42) in

PDAPP mic was decreased by 7.0 μg/g w t weight (at 15 months of ag) after 4 months of monthly inoculation with synthetic Aβ1-42 compared to sham-treated controls, and by 13.8 μg/g (18 months of age) after 7 m nths of monthly treatment. Monthly treatment with anti-Aβ antibody injections for 6 months induced an 8.9 μg/g reducti n in total cerebral Aβ1-42 in the sam mouse model at 15 months of age (Bard et al., 2000). Since Aβ1-42 represents ~90% of total Aβ species in this model (Johnson-Wood et al., 1997), the estimated decrease in total Aβ reported by Bard et al. (2000) is ~10 μg/g. Recently, intranasal Aβ immunotherapy has been reported to induce a 1.5 μg/g decrease in Aβ in PDAPP transgenic mice (Weiner et al., 2000), and a replication of the original Aβ immunization protocol (Schenk et al., 1999) failed to decrease total Aβ but did induce a marked decrease in plaque surface area in TgCRND8 transgenic mice (Janus et al., 2000).

In comparison, we found a 375 μg/g reduction in extracted total cerebral Aβ with CQ treatment in 23-month-old APP2576 mice. Although this ~50% decrease compared to sham-treated controls is proportionally less than the best reported effects of the Aβ vaccination in older mice (60% and 80% decreases in 15- and 18-month-old PDAPP mice, respectively) (Schenk et al., 1999), the absolute reduction in Aβ induced by CQ is ~30 times greater. Furthermore, the beneficial effect of CQ was achieved more rapidly with CQ (9 weeks) than with the vaccine protocol (4 and 7 months).

Lim et al. (2000) recently reported a decrease in total Aβ of 22 ng/cerebral hemisphere (untreated controls = 54.7 ng, ibuprofen treated = 32.7 ng, approximately −40%) in the 2% SDS insoluble pellet fraction of 16-month-old APP2576 mice treated for 6 months with ibuprofen 56 mg/kg/d, an antiinflammatory drug. Contrast of our results with this study is difficult since we measured Aβ in PBS soluble and insoluble fractions; however, the PBS-insoluble values for our sham-treated 23-month-old APP2576 mice were 206 ± 25 ng/hemisphere, and for CQ treated 110 ± 11 ng/hemisphere, a difference of 96 ng/hemisphere or approximately −47% ($p = 0.002$), achieved following 9 weeks of treatment. The phosphatidylinositol kinase inhibitor wortmannin has been reported to prevent Aβ accumulation (0.2 μg/g) in the APP2576 mouse model treated for 4.5 months until 8.5 months of age (Haugabook et al., 2001). No data is yet available on the effects of wortmannin on APP2576 mice of similar ages to the groups that we studied.

CQ treatment was associated with absent histological amyloid deposition in two of five 15-month-old Tg mice (Figure 2). In our experience in examining Aβ immunohistochemistry in the brains of this APP2576 model, there has been no instance where amyloid deposition is absent at 15 months of age ($n > 200$ observations). Further studies will be necessary to determine whether CQ treatment prolonged for greater than the 9 and 12 week intervals employed here might induce more instances of complete clearing of amyloid, such as those seen in the treatment of amyloid-bearing transgenic mice inoculated for 4–7 months with synth tic Aβ1-42 (Sch nk et al., 1999). In relative terms, treatment with Aβ immunotherapy or ibuprofen achieved great r proportional decreases in th surface area of Aβ immunoreactivity than the effects of CQ treatment (−25%, Figure 3D). However, direct comparison between reports that use quantitativ image capture analysis of plaque surface area is problematic for several reasons. The reports f Aβ burden in units of surface area do not mak referenc to quantitative standards that correspond to a degree of staining intensity within a dynamic range. Therefore, the surfac area of the section that is adjudged as positive for Aβ immunoreactivity is a product of where the software is instructed to set the monochromatic threshold value for a particular series of measurements. This threshold value varies from report to report, usually depending upon the nonspecific background intensity of the preparation, and the set zero is therefore arbitrary. As a result, separate studies of cortical Aβ burden in the same mouse model result in greatly different values. For example, Schenk et al. (1999) reported the mean area of cortical 3D6 Aβ immunoreactivity in sham-treated 18-month-old PDAPP mice to be 4.87%, yet the same group using the same methods subsequently reported that sham-treated younger (15-month-old) PDAPP mice have a mean cortical 3D6 Aβ immunoreactivity that is apparently much greater (19%) than the older animals (Bard et al., 2000).

Furthermore, if the monochromatic threshold is set too high, many of the samples in a treatment group will achieve readouts that are below the threshold of detection, introducing artifactually decreased variance into the data from that group (because subzero values will be read as zero, with no variance). To address this problem, we set our threshold deliberately lower so that we could appreciate the variance in the treatment (CQ) group, which may explain why we observed a ~25% decrease in plaque surface area but the decrease in extracted Aβ was nearly twice as large (Figure 3, 50%). As a result of the arbitrary setting of threshold floor values for histological Aβ immunoreactivity, the proportional changes in plaque surface area values may not correspond to the changes in extracted Aβ levels. Without a means of standardizing such data, this methodological problem invalidates proportional comparisons of plaque surface area with extracted Aβ values and also disqualifies the reference of plaque surface area changes in treated brains as a proportion of such surface area in untreated brains. Hence, we believe that the commonly used description of proportional changes in immunohistochemical plaque surface area within or between studies is an incorrect practice. Therefore, we have reported absolute changes in plaque surface area; the reduction in immunoreactive plaque surface area that we observed (4.3 μ^2/100 μ^2) was approximately the same as that reported by Schenk et al. (1999) (4.8 μ^2/100 μ^2), but additional controls would be necessary to validate such a comparison.

These methodological issues may contribute to the lack of correlation between extracted brain Aβ values and plaque surface area in human studies (McLean et al., 1999) and in the current study. However, plaque may be a qualitative feature of Aβ accumulation produced by local neurochemical interactions, and not a strict product of Aβ concentration. This would explain why levels of Aβ are elevated to the sam extent in both neocortex and basal ganglia in AD (McLean et al., 1999), yet distinct plaques do not appear in th basal ganglia.

The biochemical distinction between plaqu and nonplaqu Aβ values could b important in evaluating experimental treatment approaches In transgenic mouse models. F r example, Aβ vaccinati n has recently been reported to decrease plaque surface area but not lev ls of total extracted Aβ (Janus et al., 2000). In contrast, the inability of CQ to resolubiliz synth tic β-sheet-mediated Aβ aggregates (Figure 1A) may be compatible with CQ impacting less on plaque deposition than on diffuse Aβ deposition (Figure 3). Therefore, the two proposed therapies may be targeting different biochemical forms of Aβ. Both Janus et al. (2000) and our current study report in vivo improvements in the treated animals so that the relationship between physiological deficits and the accumulation of a specific species of Aβ is likely to be complex.

Given the caveats in making comparisons between these studies (e.g., differences in transgenic mouse models, differences in ages of cohorts, exponential accumulation of Aβ as the animals age, differences in extraction and assay procedures), it is not yet possible to draw firm conclusions about the relative potencies of CQ compared to other candidate treatment approaches. Further side-by-side comparative studies are required to achieve a true appraisal of the relative efficacies of these various treatment approaches. Nevertheless, the 375 μg/g reduction in total cerebral Aβ that was achieved with CQ is also meaningful because the concentration of total Aβ (using similar assay methods) in AD-affected neocortex is only 20–30 μg/g wet weight (Cherny et al., 1999; McLean et al., 1999). We therefore conclude from our results that CQ treatment at this dose leads to a marked interruption in cerebral Aβ accumulation that could potentially impact upon Aβ accumulation in AD, and that the dose of CQ per kilogram used in this study might exceed what would be needed for a beneficial effect in human clinical trials.

CQ treatment of the 21-month-old APP2576 mice elevated the concentration of soluble brain Aβ by 3.45 μg/g wet weight (Figure 3B). Although this is a ~50% increase in soluble Aβ levels compared to untreated animals, it represents only a ~1% rise in total Aβ levels (Figure 3C) and is overshadowed by the more profound (100-fold) decrease in insoluble Aβ (−375 μg/g, Figure 3A) leading to a net ~50% decrease in total Aβ burden. There is some concern that elevating soluble Aβ levels may contribute to pathophysiology since we (McLean et al., 1999) and others (Lue et al., 1999; Wang et al., 1999) have reported that the levels of soluble Aβ in AD c rebral tissue correlate with neuritic change, neurofibrillary tangle load, and inversely correlate with life expectancy, suggesting that soluble forms of Aβ may mediate toxicity in AD. Furthermore, toxic soluble forms of Aβ have been purified from AD-affected brain (Kuo et al., 1996). However, there was no evidence in the current study that the increase in soluble Aβ was accompanied by any adverse effects, abbreviated life span, or synaptic loss. Nontoxic soluble Aβ species are found in normal brain tissue (Cherny et al., 1999; McLean et al., 1999). Also, not all forms of Aβ are toxic even in the AD-affected brain since there is a zinc-bound form whose abundance is inversely correlated with oxidativ damage to neuropil (Cuajungco et al., 2000). It may be this form that is liberated into the soluble phase upon CQ treatment, such as wh n postmortem AD-affected brain tissu is treated with chelators (Cherny et al., 1999) lik CQ (Figure 1B). Therefore, the soluble Aβ species that is elevated f ll wing CQ treatment is either a nont xic form of Aβ or its toxicity has been attenuated by reaction with CQ.

Th increase in th ratio of soluble to total Aβ could also represent a physiological normalization since, in AD, this ratio falls (Cherny et al., 1999; McLean et al., 1999), probably due to the reaction that drives the precipitation of the peptide in the disease. The CQ-associated rise in soluble Aβ levels associated with a net decrease in total Aβ implies that the Aβ deposits are dissociating into the soluble phase. The detection of elevated soluble Aβ levels in the CQ-treated mice also suggests that APP processing is not inhibited by CQ treatment. Therefore, in contrast to APP secretase inhibitors that decrease soluble Aβ levels, the CQ treatment is expected to increase soluble Aβ levels in the process of reversing Aβ deposition. Further studies will be necessary to determine whether cerebral soluble Aβ levels will rise further or ultimately fall if Aβ deposition was abolished by CQ, say as a result of more prolonged treatment of the mice.

Although treatment with a chelating agent may be expected to deplete systemic metal levels causing adverse effects, we found no depletion of peripheral metal levels. This result is probably a reflection of the low affinity of CQ for Zn^{2+} (K_1 = 7.0) and Cu^{2+} (K_1 = 8.9) so that once these metal ions are released from the cerebral amyloid mass, the affinity of the drug is too low to lead to net metal excretion in the face of the homeostatic response to maintain systemic metal levels. This result suggests that the drug may induce remission of amyloid deposition in AD itself without necessarily depleting tissue metal levels.

The 15% elevation in soluble Zn (+9.6 μM) and Cu (+4.7 μM) levels in the brain after treatment with CQ (Table 1) is surprising since CQ treatment of nontransgenic mice (10 mg/kg/d for 20 days) significantly decreased (−25%) brain Cu and Fe levels, although it did not change Zn levels (Yassin et al., 2000). We hypothesize that the rise in cerebral Zn and Cu levels in CQ-treated APP2576 mice may reflect the tissue scavenging of Aβ coprecipitated with Cu^{2+} and Zn^{2+} that is liberated by the action of CQ; the Aβ is proteolytically degraded while the metals are stored transiently in the metallothionein pool. It is also possible that CQ treatment has adjusted metal homeostasis in the tissue by altering the turnover of Aβ and APP. Studies of APP knockout mice indicate that Aβ and/or APP could be involved in Cu, Zn, and Fe homeostasis in the cerebral cortex and peripheral tissue, as evidenced by significantly increased Cu levels, and a trend toward increased Zn and Fe levels, in these tissues (White et al., 1999). Therefore, APP2576 mice that overexpress APP may be expected to have constitutively decreased levels of these metals. If Aβ plays such a role in metal homeostasis, CQ may indirectly correct the depletion of metals in the transgenic tissue by facilitating Aβ turnover. In agreement with this interpretation of th findings that clioquinol increases copper levels in APP2576 transgenic mice by correcting a defect in copper homeostasis, we have recently found that thes mic , as well as transgenic mice expressing

the carboxy-terminal 100 amin acids of APP, both develop significantly decreased copper l vels in brain tissue as they age (C. Maynard, R.A.C., C.L.M., A.I.B., R. Cappai, and Q.X. Li, unpublished data). This implicates the turnover of Aβ itself in sustaining brain copper lev ls.

CQ-treated animals did significantly better than sham-treated controls in an index of general impairment and also maintenance of body weight (Figures 4A and 4C). Despite being a crude instrument, the impairment index that we used detected a highly significant improvement in CQ-treated versus sham-treated mice. Further studies with more precise cognitive tests in younger animals capable of maze tasks are needed to determine whether this effect is due to systemic health effects or specific cognitive effects. Since clearance of Aβ accumulation (by vaccination) is associated with improved performance on cognitive testing of AD transgenic mice, we predict that the clearance of Aβ by CQ will also be accompanied by gains in cognitive performance. Nevertheless, our findings that the general well being of the animals improved with treatment raise the concern that studies of candidate treatments in transgenic mice will need to control for general health effects when testing performance in maze tasks. Improved maze performance may be a reflection of improved general health or alertness, and not necessarily of improved selective memory function. The blinded index that we employed is a facile and inexpensive means of introducing such a control.

We have shown that treatment with a lipophilic Zn/Cu chelator that crosses the blood-brain barrier attenuates Aβ deposition in a mouse model for AD, encouraging us to translate this novel approach into the clinical setting. Our results may explain how desferrioxamine, a parenteral chelator with affinities for Cu^{2+}, Zn^{2+}, Fe^{3+}, and Al^{3+}, may have acted to inhibit the progression of AD in a clinical trial (Crapper McLachlan et al., 1991).

Our results suggest that CQ may be suitable for testing in clinical trials in AD patients. The recommended dose of CQ when it was prescribed as an antiamebic was 500 mg 3–4 times/day (20 mg/kg/d), which is a weight-normalized dosage level that achieved inhibition of Aβ deposition in our current studies. A caveat in the clinical use of CQ is that it has been associated with subacute myelo-optic neuropathy (SMON), an uncommon neurological syndrome largely confined to Japan (Tsubaki et al., 1971). A causal relationship between CQ and SMON was never proven (Clifford Rose and Gawel, 1984; Meade, 1975), but the relatively low benefit of the drug balanced against the postulated risk of such a serious side effect led to its worldwide withdrawal in the early 1970s. Our current findings suggest that a reexamination of this drug and its side effects may be warranted.

CQ was used extensively for 20 years before the first case of SMON was described, and before its retirement, the drug was used for 500 million patient days as an antibiotic with a very favorable safety profile. SMON resembles an accelerated form of subacute combined degeneration due to vitamin B12 deficiency, and administration of CQ to normal mice has been reported to deplet brain and serum levels of vitamin B12 (Yassin et al., 2000). Nevertheless, no clear relationship has been identified between CQ dose and the risk for SMON in humans. Six cases of encephalopathy (but not SMON) induced by acut overd ses in excess f 7.5 g have been reported (Baumgartner et al., 1979). Conversely, 25% of patients with SMON (in a sampl of 2465 from Japan) had n ver taken CQ (Naka et al., 1973). In the 1960s, the per capita consumption of CQ was higher in several other countries than the consumption in Japan. However, until 1975 there were 10,000 cases of SMON in Japan, while there were only 220 cases identified in the rest of the world. Therefore, it is possible that local demographic factors prevalent in Japan at the time may have predisposed this population to develop SMON. One possibility is that the Japanese were endemically B12 deficient as a consequence of their diet in the post-war years and that this was the predisposing factor to SMON. CQ was commonly used to treat gastrointestinal symptoms in an unregulated manner in Japan in that era, and SMON usually begins with symptoms of abdominal pain and diarrhea, therefore overdosing in a B12-deficient population may have exaggerated the incidence of SMON in Japan. In light of this possible explanation for the association of CQ with SMON, coadministration of vitamin B12 is part of the phase two clinical trial that is currently in progress (C.L.M. and A.I.B., unpublished data). A completed phase one clinical trial of CQ with B12 supplementation in AD patients revealed no adverse systemic, neurological, or cognitive effects (C.G. Gottfries and M.X., unpublished data). Our current findings indicate that CQ and its derivatives, or other hydrophobic chelators, merit further investigation for their therapeutic utility in the prevention and treatment of AD.

Experimental Procedures

Effects of Chelators on Metal-Induced Aβ Aggregation

Aβ1-40 (10 ng in 200 μl) aggregation was induced by incubation (30 min, RT) with $ZnCl_2$ (25 μM in TBS, pH 7.4), $CuCl_2$ (5 μM in TBS, pH 6.8), or acidic conditions (pH 5.5, MES buffered saline). Aggregates were transferred to a 0.2 μ nylon membrane by filtration using a 96-well ELIFA apparatus (Pierce). The aggregates were then washed (200 μl/well) with TBS alone, TBS containing 2 μM EDTA, or TBS with 2 μM CQ. The membrane was fixed, probed with the anti-Aβ monoclonal antibody 6E10 (Senetek), and developed for exposure to ECL film. Quantification of retained, aggregated Aβ was performed by densitometry, calibrated against known amounts of the peptide.

NMR Spectroscopy

Six hundred megahertz 1H NMR spectra were taken of 0.3–1 mM Aβ peptides in 50 mM phosphate buffer (pH 6.9) 100 mM NaCl, 10% ^2H_2O at 271°K, using a Bruker DRX-600 instrument. CQ was first dissolved in DMSO and then added at 800 μM to ensure that the solution was saturated with soluble CQ. The maximal soluble concentration of CQ prepared in this manner was determined to be ~100 μM, so an aqueous suspension was formed. NMR peaks due to soluble CQ were detectable despite the presence of the majority of the CQ being in suspension.

Mice Studies

All mice were housed according to standard animal care protocols, fed *ad libitum*, and maintained in a pathogen-free environment at the MGH Neuroscience Center. The transgenic status of all animals was confirmed by PCR of tail snips, using the 3′UTR of the hamster cosmid PrP vector as a hybridization probe, and for overexpression on the APP transgene by Western blot (22C11) of postmortem brain tissue. The colony was maintained by Charles River Laboratories by crossing female Tg (HuAPP695.SWE)2576 with B6SJLF1/J males (Jackson Labs). The animals were randomized for therapy trials, coded, and the operators and data analysts remained double blind

to which treatment (or placebo) they received, until the code was broken at the completion of data collection. The treated animals were delivered, the chelator dissolved in 0.05% carboxymethylcellulose (Sigma) by daily gavage, and the untreated controls were gavaged with the placebo vehicle alone. The mice did not receive B12 supplementation.

The choice of dose of CQ for these studies was based upon pilot studies and a review of the literature. Preparatory studies determined that 4-month-old nontransgenic mice tolerated CQ at 40 mg/kg/day for 7 days with no apparent adverse effects. An SMON-like syndrome can be induced in dogs by sustained doses of CQ (>150 mg/kg/day), but mice and other rodents are far less susceptible to this syndrome (Tateishi and Otsuki, 1975). To minimize the chance of neurological side effects in our studies, we chose doses of 30 mg/kg/d or less for periods no greater than 12 weeks.

Mouse Data Collection

In the study of 21-month-old APP2576 mice, the animals were examined daily by a blinded operator, and a measurement of each animal's general behavior in its cage was taken by observation based upon a subjective 5 point rating scale, where 5 is alert, grooming, normal withdrawal response upon handling, and no obvious motor abnormality; 4 is either distressed or lethargic, not grooming, lost withdrawal response upon handling, but no motor abnormality; 3 is periodic obvious motor abnormality (paresis, spinning, tremor, rigidity); 2 is persistent motor abnormality or cachexia; and 1 is moribund. The animals were also weighed at intervals. Equality of survival distributions was statistically tested by log-rank analysis. All statistical analyses used Systat 9.0 (SPSS, Inc.). At the completion of all studies, the animals were anesthetized, a blood sample obtained, cardiac-perfused with cold saline, and the brain and peripheral organs removed. The left cerebral hemisphere was fixed in 4% paraformaldehyde, and the right hemisphere (without cerebellum) and remaining tissues were weighed and snap frozen in liquid nitrogen.

Aβ, APP, Synaptophysin, and GFAP Analyses

Snap-frozen tissues were thawed and homogenized in PBS (pH 7.4, 2 ml) and centrifuged at 100,000 × g for 30 min. Aβ in the supernatants (soluble), the pellet, in an aliquot of homogenate (total), and in serum, was quantified by Western blot using WO2, an anti-Aβ monoclonal antibody that detects all forms of full-length Aβ, calibrated with known quantities of synthetic Aβ, as previously described (Cherny et al., 1999; McLean et al., 1999). APP was quantified by Western blot from the same samples using 22C11 (Boehringer), which detects both the transgene-expressed human APP as well as the endogenous mouse APP, and using recombinant APP standards. This antibody is directed to the amino terminus of APP (Hilbich et al., 1993) and cannot differentiate between soluble and full-length APP; therefore, soluble and full-length APP levels were respectively measured from the supernatant and SDS- extracted pellet fractions of the cerebral homogenates after ultracentrifugation. Synaptophysin levels were measured in protein-normalized samples of total brain homogenate by Western blot (monoclonal antibody SY38, Boehringer), and relative values are reported in arbitrary absorbence units (U/g protein) after computer-assisted densitometric analysis of the films and ascertainment that the signals were in linear dynamic range (described in Cherny et al., 1999).

Formic acid extraction of tissue, while efficient, is problematic since the procedure chemically modifies Aβ (formication) and also requires the samples to be laboriously neutralized before PAGE analysis. For the measurement of PBS-insoluble Aβ in the brain homogenates, it was determined that the extraction of Aβ from the brain tissue by 70% formic acid (FA) treatment was no more efficient that extraction by 8% SDS sample buffer alone. The pellets remaining following PBS extraction and ultracentrifugation were resuspended 1:1000 (w/v) in PBS and aliquots were dissolved by extensive boiling in 8% SDS sample buffer containing 10% mercaptoethanol prior to separation on PAGE. No immunoreactive Aβ remained following this procedure and this was confirmed by examining subsequent FA extracts by Western analysis. The adoption of such a solubilization protocol that avoids the use of FA was developed in light of publications that observed that sequential extractions of human AD brain in various water based buffers or 10% SDS yielded further solubilization of Aβ from the formic acid "insoluble" pellet fraction (Harigaya et al., 1995; Tamaoka et al., 1994).

Furthermore, the Roher laboratory recently published that the brain Aβ deposits in the APP23 transgenic mouse model for Alzheimer's disease can also be fully extracted with SDS and, unlike human brain Aβ deposits, do not require FA treatment to be liberated (Kuo et al., 2001). Our own methodological experiments confirmed a similar observation in the brains of APP2576 mice. We found that in mice with advanced amyloid pathology (18–20 months), extraction of the brain homogenate into 8% SDS was 100% efficient, leaving no FA-extractable Aβ. This suggests, in agreement with Kuo et al. (2001), that the Aβ deposits in the transgenic mouse model contain less oxidative modifications than the Aβ that comprises amyloid in the human pathology and hence is more readily extracted by detergents. Therefore, apart from the pilot study of chelation treatment, brain homogenate samples were extracted into SDS sample buffer for Western blot.

Histological sections of whole brain were prepared and the proportional surface area of amyloid plaques estimated by computer-assisted immunohistochemical quantification (using monoclonal antibody 1E8), as described previously (McLean et al., 1999). Sections of the hippocampus were also stained with a monoclonal antibody against glial fibrillary acidic protein (DAKO) and the number of cells in the pyramidal layer staining positively per high-powered field (hpf) was determined (n = 3 fields, n = 3 sections). The operator remained blind to the CQ treatment status of the tissue. Data from the treated and untreated animal groups were analyzed by two-tailed t test.

Metal Quantification

Aliquots were taken from the supernatant samples of the tissue homogenates and diluted in 1% HNO_3. The pellets were freeze-dried and digested in 300 μl HNO_3, followed by 300 μl of H_2O_2 at 70°C, and further diluted in 1% HNO_3 for analysis by inductively coupled plasma mass spectrometry (ICP-MS). ICP-MS was performed using an Ultramass 700 (Varian, Vic., Australia) in peak-hopping mode with spacing at 0.100 AMU, 1 point per peak, 50 scans per replicate, 3 replicates per sample. Plasma flow was 15 L/min with auxiliary flow 1.5 L/min. RF power was 1.2 kW. Sample was introduced using a glass nebulizer at a flow of 0.88 L/min. The apparatus was calibrated using a 1% HNO_3 solution containing Cu and Zn at 5, 10, 50, and 100 ppb with Y89 the internal standard for all isotopes of Cu and Zn. Metal values are mean μg/g wet weight of the original tissue sample. Two-tailed t test assuming unequal variances was performed on differences between the mean values from the untreated (C) compared to clioquinol-treated (CQ) samples.

Acknowledgments

This work was supported by funds from Prana Biotechnology Ltd, the National Institute on Aging, the American Health Assistance Foundation, the Percy Baxter Charitable Trust, and the NH&MRC. We thank Karen Hsiao Ashe for the APP2576 mice, Margaret Smith for assistance with genotyping, Maarten van den Buuse and Andrew McKinnon for statistical advice, and Craig Ritchie and Stephen MacFarlane for helpful discussion. The following authors are minor equity holders in Prana Biotechnology Ltd: R.A.C., K.J.B., R.E.T., C.L.M., and A.I.B.

Received December 11, 2000; revised March 29, 2001.

References

Atwood, C.S., Moir, R.D., Huang, X., Bacarra, N.M.E., Scarpa, R.C., Romano, D.M., Hartshorn, M.A., Tanzi, R.E., and Bush, A.I. (1998). Dramatic aggregation of Alzheimer Aβ by Cu(II) is induced by conditions representing physiological acidosis. J. Biol. Chem. 273, 12817–12826.

Atwood, C.S., Scarpa, R.C., Huang, X., Moir, R.D., Jones, W.D., Fairlie, D.P., Tanzi, R.E., and Bush, A.I. (2000). Characterization of copper interactions with Alzheimer Aβ peptides—identification of an attomolar affinity copper binding site on Aβ1–42. J. Neurochem. 75, 1219–1233.

Bard, F., Cannon, C., Barbour, R., Burke, R.L., Games, D., Grajeda,

H., Guido, T., Hu, K., Huang, J., Johnson-Wood, K., et al. (2000). Peripherally administered antibodies against amyloid beta-peptide enter the central nervous system and reduce pathology in a mouse model of Alzheimer disease. Nat. Med. *6*, 916–919.

Baumgartner, G., Gawel, M.J., Kaeser, H.E., Pallis, C.A., Rose, F.C., Schaumburg, H.H., Thomas, P.K., and Wadia, N.H. (1979). Neurotoxicity of halogenated hydroxyquinolines: clinical analysis of cases reported outside Japan. J. Neurol. Neurosurg. Psychiatry *42*, 1073–1083.

Bush, A.I., Pettingell, W.H., Jr., Paradis, M.D., and Tanzi, R.E. (1994a). Modulation of Aβ adhesiveness and secretase site cleavage by zinc. J. Biol. Chem. *269*, 12152–12158.

Bush, A.I., Pettingell, W.H., Multhaup, G., Paradis, M.D., Vonsattel, J.P., Gusella, J.F., Beyreuther, K., Masters, C.L., and Tanzi, R.E. (1994b). Rapid induction of Alzheimer Aβ amyloid formation by zinc. Science *265*, 1464–1467.

Bush, A.I., Huang, X., and Fairlie, D.P. (1999). The possible origin of free radicals from amyloid β peptides in Alzheimer's disease. Neurobiol. Aging *20*, 335–337.

Cherny, R.A., Legg, J.T., McLean, C.A., Fairlie, D., Huang, X., Atwood, C.S., Beyreuther, K., Tanzi, R.E., Masters, C.L., and Bush, A.I. (1999). Aqueous dissolution of Alzheimer's disease Aβ amyloid deposits by biometal depletion. J. Biol. Chem. *274*, 23223–23228.

Clifford Rose, F., and Gawel, M. (1984). Clioquinol neurotoxicity: an overview. Acta Neurol. Scand. Suppl. *100*, 137–145.

Crapper McLachlan, D.R., Dalton, A.J., Kruck, T.P.A., Bell, M.Y., Smith, W.L., Kalow, W., and Andrews, D.F. (1991). Intramuscular desferrioxamine in patients with Alzheimer's disease. Lancet *337*, 1304–1308.

Cuajungco, M.P., Goldstein, L.E., Nunomura, A., Smith, M.A., Lim, J.T., Atwood, C.S., Huang, X., Farrag, Y.W., Perry, G., and Bush, A.I. (2000). Evidence that the beta-amyloid plaques of Alzheimer's disease represent the redox-silencing and entombment of Aβ by zinc. J. Biol. Chem. *275*, 19439–19442.

Harigaya, Y., Shoji, M., Kawarabayashi, T., Kanai, M., Nakamura, T., Iizuka, T., Igeta, Y., Saido, T.C., Sahara, N., Mori, H., et al. (1995). Modified amyloid β protein ending at 42 or 40 with different solubility accumulates in the brain of Alzheimer's disease. Biochem. Biophys. Res. Commun. *211*, 1015–1022.

Haugabook, S.J., Le, T., Yager, D., Zenk, B., Healy, B.M., Eckman, E.A., Prada, C., Younkin, L., Murphy, P., Pinnix, I., et al. (2001). Reduction of Aβ accumulation in the Tg2576 animal model of Alzheimer's disease after oral administration of the phosphatidyl-inositol kinase inhibitor wortmannin. FASEB J. *15*, 16–18.

Hilbich, C., Monning, U., Grund, C., Masters, C.L., and Beyreuther, K. (1993). Amyloid-like properties of peptides flanking the epitope of amyloid precursor protein-specific monoclonal antibody 22C11. J. Biol. Chem. *268*, 26571–26577.

Hsiao, K., Chapman, P., Nilsen, S., Eckman, C., Harigaya, Y., Younkin, S., Yang, F., and Cole, G. (1996). Correlative memory deficits, Aβ elevation, and amyloid plaques in transgenic mice. Science *274*, 99–102.

Huang, X., Atwood, C.S., Moir, R.D., Hartshorn, M.A., Vonsattel, J.-P., Tanzi, R.E., and Bush, A.I. (1997). Zinc-induced Alzheimer's Aβ1-40 aggregation is mediated by conformational factors. J. Biol. Chem. *272*, 26464–26470.

Huang, X., Atwood, C.S., Hartshorn, M.A., Multhaup, G., Goldstein, L.E., Scarpa, R.C., Cuajungco, M.P., Gray, D.N., Lim, J., Moir, R.D., et al. (1999a). The Aβ peptide of Alzheimer's disease directly produces hydrogen peroxide through metal ion reduction. Biochemistry *38*, 7609–7616.

Huang, X., Cuajungco, M.P., Atwood, C.S., Hartshorn, M.A., Tyndall, J., Hanson, G.R., Stokes, K.C., Leopold, M., Multhaup, G., Goldstein, L.E., et al. (1999b). Cu(II) potentiation of Alzheimer Aβ neurotoxicity: correlation with cell-free hydrogen peroxide production and metal reduction. J. Biol. Chem. *274*, 37111–37116.

Ida, N., Hartmann, T., Pantel, J., Schroder, J., Zerfass, R., Forstl, H., Sandbrink, R., Masters, C.L., and Beyreuther, K. (1996). Analysis of heterogeneous A4 peptides in human cerebrospinal fluid and blood by a newly developed sensitive Western blot assay. J. Biol. Chem. *271*, 22908–22914.

Janus, C., Pearson, J., McLaurin, J., Mathews, P.M., Jiang, Y., Schmidt, S.D., Chishti, M.A., Horne, P., Heslin, D., French, J., et al. (2000). A beta peptide immunization reduces behavioural impairment and plaques in a model of Alzheimer's disease. Nature *408*, 979–982.

Johnson-Wood, K., Lee, M., Motter, R., Hu, K., Gordon, G., Barbour, R., Khan, K., Gordon, M., Tan, H., Games, D., et al. (1997). Amyloid precursor protein processing and Aβ42 deposition in a transgenic mouse model of Alzheimer disease. Proc. Natl. Acad. Sci. USA *94*, 1550–1555.

Kidani, Y., Naga, S., and Koike, H. (1974). Mass spectrophotometry of 5-chloro-7iodo-8-quinolinol metal chelates. Jap. Analyst *23*, 1375–1378.

Kotaki, H., Yamamura, Y., Tanimura, Y., Saitoh, Y., Nakagawa, F., and Tamura, Z. (1983). Intestinal absorption and metabolism of clioquinol in the rat. J. Pharm. Dyn. *6*, 881–887.

Kuo, Y.M., Emmerling, M.R., Vigo-Pelfrey, C., Kasunic, T.C., Kirkpatrick, J.B., Murdoch, G.H., Ball, M.J., and Roher, A.E. (1996). Water-soluble Aβ (N-40, N-42) oligomers in normal and Alzheimer disease brains. J. Biol. Chem. *271*, 4077–4081.

Kuo, Y.M., Kokjohn, T.A., Beach, T.G., Sue, L.I., Brune, D., Lopez, J.C., Kalback, W.M., Abramowski, D., Sturchler-Pierrat, C., Staufenbiel, M., and Roher, A.E. (2001). Comparative analysis of Aβ chemical structure and amyloid plaque morphology of transgenic mice and Alzheimer disease brains. J. Biol. Chem. *276*, 12991–12998.

Lee, J.-Y., Mook-Jung, I., and Koh, J.-Y. (1999). Histochemically reactive zinc in plaques of the Swedish mutant beta-amyloid precursor protein transgenic mice. J. Neurosci. *19; RC10*, 1–5.

Lim, G.P., Yang, F., Chu, T., Chen, P., Beech, W., Teter, B., Tran, T., Ubeda, O., Ashe, K.H., Frautschy, S.A., and Cole, G.M. (2000). Ibuprofen suppresses plaque pathology and inflammation in a mouse model for Alzheimer's disease. J. Neurosci. *20*, 5709–5714.

Lovell, M.A., Robertson, J.D., Teesdale, W.J., Campbell, J.L., and Markesbery, W.R. (1998). Copper, iron and zinc in Alzheimer's disease senile plaques. J. Neurol. Sci. *158*, 47–52.

Lue, L.F., Kuo, Y.M., Roher, A.E., Brachova, L., Shen, Y., Sue, L., Beach, T., Kurth, J.H., Rydel, R.E., and Rogers, J. (1999). Soluble amyloid beta peptide concentration as a predictor of synaptic change in Alzheimer's disease. Am. J. Pathol. *155*, 853–862.

McLean, C.A., Cherny, R.A., Fraser, F.W., Fuller, S.J., Smith, M.J., Beyreuther, K., Bush, A.I., and Masters, C.L. (1999). Soluble pool of Aβ amyloid as a determinant of severity of neurodegeneration in Alzheimer's Disease. Ann. Neurol. *46*, 860–866.

Meade, T.W. (1975). Subacute myelo-optic neuropathy and clioquinol. An epidemiological case-history for diagnosis. Br. J. Prev. Soc. Med. *29*, 157–169.

Moir, R.D., Atwood, C.S., Romano, D.M., Laurans, M.H., Huang, X., Bush, A.I., Smith, J.D., and Tanzi, R.E. (1999). Differential effects of apolipoprotein E isoforms on metal-induced aggregation of Aβ using physiological concentrations. Biochemistry *38*, 4595–4603.

Nakae, K., Yamamoto, S., Shigematsu, I., and Kono, R. (1973). Relation between subacute myelo-optic neuropathy (S.M.O.N.) and clioquinol: nationwide survey. Lancet *1*, 171–173.

Padmanabhan, G., Becue, I., and Smith, J.A. (1989). Clioquinol. In Analytical Profiles of Drug Substances, E. Klauss, and E. Florey, eds. (New York: Academic Press), pp. 57–90.

Richards, D.A. (1971). Prophylactic value of clioquinol against travellers' diarrhea. Lancet *1*, 44–45.

Schenk, D., Barbour, R., Dunn, W., Gordon, G., Grajeda, H., Guido, T., Hu, K., Huang, J., Johnson-Wood, K., Khan, K., et al. (1999). Immunization with amyloid-beta attenuates Alzheimer-disease-like pathology in the PDAPP mouse. Nature *400*, 173–177.

Shiraki, H. (1979). Handbook of Clinical Neurology, Vol. 37 (Amsterdam: Elsevier/North-Holland Biomedical Press).

Smith, M.A., Harris, P.L.R., Sayre, L.M., and Perry, G. (1997). Iron accumulation in Alzheimer's disease is a source of redox-generated free radicals. Proc. Natl. Acad. Sci. USA *94*, 9866–9868.

Soto, C., Kindy, M.S., Baumann, M., and Frangione, B. (1996). Inhibition of Alzheimer's amyloidosis by peptides that prevent β-sheet conformation. Biochem. Biophys. Res. Commun. *226*, 672–680.

Suh, S.W., Jensen, K.B., Jensen, M.S., Silva, D.S., Kesslak, J.P., Danscher, G., and Frederickson, C.J. (2000). Histological evidence implicating zinc in Alzheimer's disease. Brain Res. *852*, 274–278.

Tamaoka, A., Kondo, T., Odaka, A., Sahara, N., Sawamura, N., Ozawa, K., Suzuki, N., Shoji, S., and Mori, H. (1994). Biochemical evidence for the long-tail form (Aβ 1–42/43) of amyloid β protein as a seed molecule in cerebral deposits of Alzheimer's disease. Biochem. Biophys. Res. Commun. *205*, 834–842.

Tateishi, J., and Otsuki, S. (1975). Experimental reproduction of SMON in animals by prolonged administration of clioquinol: clinicopathological findings. Jpn. J. Med. Sci. Biol. *28*, 165–186.

Toyokura, Y., Takasu, T., and Matsuoka, O. (1975). Experimental studies utilizing radionuclide-labeled clioquinol as tracer in vivo. Jpn. J. Med. Sci. Biol. *28*, 79–86.

Tsubaki, T., Honma, Y., and Hoshi, M. (1971). Neurological syndrome associated with clioquinol. Lancet *1*, 696–697.

Wang, J., Dickson, D.W., Trojanowski, J.Q., and Lee, V.M. (1999). The levels of soluble versus insoluble brain Abeta distinguish Alzheimer's disease from normal and pathologic aging. Exp. Neurol. *158*, 328–337.

Weiner, H.L., Lemere, C.A., Maron, R., Spooner, E.T., Grenfell, T.J., Mori, C., Issazadeh, S., Hancock, W.W., and Selkoe, D.J. (2000). Nasal administration of amyloid-beta peptide decreases cerebral amyloid burden in a mouse model of Alzheimer's disease. Ann. Neurol. *48*, 567–579.

White, A.R., Reyes, R., Mercer, J.F., Camakaris, J., Zheng, H., Bush, A.I., Multhaup, G., Beyreuther, K., Masters, C.L., and Cappai, R. (1999). Copper levels are increased in the cerebral cortex and liver of APP and APLP2 knockout mice. Brain Res. *842*, 439–444.

Wood, S.J., Maleeff, B., Hart, T., and Wetzel, R. (1996). Physical, morphological and functional differences between ph 5.8 and 7.4 aggregates of the Alzheimer's amyloid peptide Aβ. J. Mol. Biol. *256*, 870–877.

Yassin, M.S., Ekblom, J., Xilinas, M., Gottfries, C.G., and Oreland, L. (2000). Changes in uptake of vitamin B(12) and trace metals in brains of mice treated with clioquinol. J. Neurol. Sci. *173*, 40–44.